

## ORIGINAL ARTICLE

## c-KIT and PDGFRA in breast phyllodes tumours: overexpression without mutations?

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**Aim:** To study the immunoexpression and mutational status of c-KIT and PDGFRA in a series of benign and malignant phyllodes tumours of the breast.

**Material/methods:** Nineteen phyllodes tumours (13 benign and six malignant) were analysed by immunohistochemistry for the expression of c-KIT and PDGFRA. Direct sequencing of exons 9, 11, 13, and 17 of the c-KIT gene and exons 12 and 18 of PDGFRA was performed to check the mutational status of these two genes.

**Results:** c-KIT expression was found in 12 of the 19 cases (six of the 13 benign cases and all six malignant ones) and PDGFRA expression was seen in two of the 19 cases (one benign and one malignant case); the 2415 C>T alteration in exon 17 of the c-KIT gene was found in two cases (both benign); the intronic insertion IVS17-50insT and the 2866 G>T alteration in the coding region of exon 18 of the PDGFRA gene were also found in two cases (one malignant and one benign). However, the activating mutations described for these genes in gastrointestinal stromal tumours were not present.

**Conclusion:** c-KIT expression is a frequent finding in phyllodes tumours, particularly in malignant cases; however, no activating mutations similar to those described for gastrointestinal stromal tumours were found. The PDGFRA does not seem to be an alternative pathway to tumour development in phyllodes tumours because neither expression nor activating mutations were noteworthy.

c-KIT and PDGFRA (platelet derived growth factor receptor A) belong to the platelet derived growth factor receptor subfamily of tyrosine kinase receptors. c-KIT is a protooncogene located at 4q12, which spans 21 exons and encodes a protein that comprises an extracellular domain with five immunoglobulin-like repeats, a transmembrane domain, a juxtamembrane domain, and a tyrosine kinase domain.<sup>1</sup> The c-KIT ligand is known as stem cell factor,<sup>2,3</sup> and the complex formed by the interaction of the receptor with its ligand is of paramount importance for haemopoiesis, melanogenesis, and gametogenesis, in addition to the development of the interstitial cells of Cajal.<sup>4,5</sup> PDGFRA is a gene that spans 23 exons and is located at the same locus as c-KIT. It encodes a transmembrane protein composed of five immunoglobulin-like domains in the extracellular region, a transmembrane domain, an ATP binding site, and a hydrophilic kinase insert domain in the intracellular portion.<sup>6</sup> The PDGFRA ligand is platelet derived growth factor and the interaction of the receptor and its ligand results in a cascade of events that leads to cell proliferation and several other crucial processes. Ligand binding to the extracellular immunoglobulin-like domains of these proteins induces the receptors to form dimers, thereby activating the intrinsic tyrosine kinase activity of these molecules.

"Patients with gastrointestinal stromal tumours have been treated successfully with imatinib mesylate"

These two genes have received much attention in the medical literature over the past few years because ligand independent constitutive activation as a result of activating mutations in these receptors can be blocked by means of a tyrosine kinase inhibitor known as imatinib mesylate (formerly STI571; now known as Gleevec in the USA and Glivec in Europe; Novartis, Basel, Switzerland).

Overexpression of c-KIT is a hallmark<sup>7,8</sup> of gastrointestinal stromal tumours (GISTs), which are the most common mesenchymal tumours of the gastrointestinal tract. Most of these tumours harbour activating mutations, mainly clustered in exons 9, 11, 13, and 17.<sup>9</sup> GISTs that lack c-KIT mutations have intragenic activating mutations in PDGFRA.<sup>10</sup> Patients with GISTs have been treated successfully with imatinib mesylate.

Phyllodes tumours are rare fibroepithelial tumours of the breast, of which the spindle cell component bears some similarity to GISTs—both are composed of neoplastic CD34 positive stromal cells, have high recurrence rates, and the malignant cases do not respond to standard chemotherapy. There have been few studies that have analysed the expression and mutational status of c-KIT in phyllodes tumours. Similarly, to the best of our knowledge, there are no reports on the distribution of PDGFRA or the presence of PDGFRA mutations in phyllodes tumours.

The aim of the present study was: (1) to evaluate the immunohistochemical expression of c-KIT and PDGFRA in 19 cases of phyllodes tumours (13 benign and six malignant) and (2) to search for activating gene mutations in exons 9, 11, 13, and 17 of the c-KIT gene and exons 12 and 18 of the PDGFRA gene by means of direct sequencing. We hypothesised that if phyllodes tumours harbour activating mutations of one of these two genes, then patients with these tumours may benefit from treatment with imatinib mesylate.

## METHODS

## Tissue material

Nineteen surgical specimens (13 benign and six malignant) were received fresh in the pathology laboratory. The specimens were placed into phosphate buffered formalin and were

**Abbreviations:** GIST, gastrointestinal stromal tumour; PCR, polymerase chain reaction; PDGFRA, platelet derived growth factor receptor A

**Table 1** Primer sequence annealing temperatures and fragment length for the exons of c-KIT

Exon	Primer sequence (5'→3')	Annealing temperature	Fragment length (bp)
9	F: AGC CAG GGC TTT TGT TTT CT R: CAG AGC CTA AAC ATC CCC TTA	56°C	267
11	F: CCA GAG TGC TCT AAT GAC TG R: ACC CAA AAA GGT GAC ATG GA	56°C	236
13	F: CAT CAG TTT GCC AGT TGT GC R: ACA CGG CTT TAC CTC CAA TG	56°C	174
17	F: GGT TTT CTT TTC TCC TCC AAC C R: GGA TTT ACA TTA TGA AAG TCA CAG G	56°C	246

F, forward primer; R, reverse primer.

fixed for 24 hours. Representative blocks of tumour were taken, embedded in paraffin wax, cut, and stained with haematoxylin and eosin. Histopathological classification of the tumours was performed on the basis of stromal cellularity, cellular pleomorphism, mitotic activity, stromal distribution, and margin appearance (infiltrating or pushing), as described previously.<sup>11</sup>

In brief, malignant tumours were defined as having noticeable cellular atypia, high mitotic activity (more than 10 mitoses/10 high power fields), stromal cellularity and overgrowth (pronounced stromal overgrowth), and a widely infiltrating margin.

### Immunohistochemistry

The formalin fixed, paraffin wax embedded breast tissues were immunostained for c-KIT and PDGFRA using standard methods. Automated immunohistochemistry was carried out (LabVision Autostainer LV-1; LabVision Corp, Fremont, California, USA) according to the streptavidin-biotin-peroxidase technique described elsewhere,<sup>12,13</sup> including antigen retrieval methods with EDTA (EDTA buffer; LabVision Corp) for 30 minutes with wet heat at 98°C. Briefly, after washing with tap water and blockage of peroxidase (five minutes), the slides were incubated with the primary antibody for c-KIT (c-KIT mouse monoclonal antibody, clone T-595; 1/60 dilution; Novocastra, Newcastle upon Tyne, UK) or PDGFRA (PDGFRA rabbit polyclonal antibody, RB-9027-P; 1/200 dilution; LabVision Corp) for 30 minutes, followed

by detection with a labelled streptavidin-biotin immunoenzymatic antigen system (Ultravision large volume detection system, antipolyvalent horseradish peroxidase; LabVision Corp). Positive and negative controls were included in each slide run. A case of GIST with a previously characterised c-KIT gene mutation and c-KIT overexpression detected by standard immunohistochemistry methods was used as positive control. Moreover, mast cells were also used as internal positive controls for c-KIT in each slide. For PDGFRA, cutaneous mucosal transition of the anal region—medium calibre vessels in general with a muscular layer—were used as positive controls. Whenever vessels were seen in the phyllodes tumours they were used as internal positive controls. c-KIT and PDGFRA immunoreactivity was assessed as described previously.<sup>14</sup> Sections were scored semiquantitatively, in both the epithelial and stromal components, as follows: 0, 0% of cells positive; 1+, 0–25% positive; 2+, 25–50% positive; and 3+, 50–100% positive. Samples that scored 0 and 1+ were considered negative for c-KIT or PDGFRA expression and those that scored 2+ and 3+ were considered positive. The sections were scored by two pathologists and in none of the cases was there disagreement between them in the interpretation of the staining.

### DNA extraction

The stromal component of the tumours was microdissected with a sterile scalpel under a stereomicroscope to avoid

**Table 2** Summary of immunohistochemistry and mutational status for c-KIT and PDGFRA

Specimen	Histology	c-KIT epithelial staining	c-KIT stromal staining	PDGFRA epithelial staining	PDGFRA stromal staining	Mutational status
1	Malignant	+	+++	–	–	Normal
2	Malignant	+	++	–	+	Normal
3	Malignant	–	+++	–	–	IVS17-50insT 2866 G>T
4	Malignant	–	+++	–	–	Normal
5	Malignant	+	++	–	–	Normal
6	Malignant	+	+++	–	++	Normal
7	Benign	++	++	–	–	Normal
8	Benign	++	+++	–	–	Normal
9	Benign	++	+++	–	–	IVS17-50insT 2866 G>T 2415 C>T
10	Benign	–	–	–	–	Normal
11	Benign	++	+++	–	–	2415 C>T
12	Benign	–	–	–	++	Normal
13	Benign	+++	–	–	+	Normal
14	Benign	++	–	–	–	Normal
15	Benign	–	–	–	–	Normal
16	Benign	–	–	–	–	Normal
17	Benign	++	–	–	–	IVS17-50insT 2866 G>T
18	Benign	+++	++	–	–	Normal
19	Benign	++	+++	–	–	Normal

**Table 3** Contingency table for c-KIT and PDGFRA

Cases	c-KIT (stromal staining)		c-KIT (epithelial staining)		PDGFRA (stromal staining)	
	Positive	Negative	Positive	Negative	Positive	Negative
Benign (n = 13)	6 (46.15%)	7 (53.85%)	9 (69.23%)	4 (30.77%)	1 (7.69%)	12 (92.31)
Malignant (n = 6)	6 (100%)	0 (0%)	0 (0%)	6 (100%)	1 (16.7%)	5 (83.3%)
p Value	0.024		0.005		0.796	

Values <0.05 were considered significant.

contamination with non-neoplastic tissues and the epithelial component. DNA extraction was performed using the NucleoSpin tissue kit for paraffin wax embedded blocks (Macherey-Nagel, Düren, Germany).

### Polymerase chain reactions

Polymerase chain reaction (PCR) primers were designed to amplify exons 9, 11, 13, and 17 of the c-KIT gene and also for amplification of exons 12 and 18 of the PDGFRA gene. The primer sequences for c-KIT were adapted from a previous study<sup>15</sup> (table 1), and for PDGFRA they were as described elsewhere.<sup>10</sup> PCR was carried out using 250 ng of DNA, 1× PCR buffer (Amersham Biosciences, Piscataway, New Jersey, USA), 200µM of each dNTP (Amersham Biosciences), 10pmol of each primer, and 1 U of Taq DNA polymerase (Amersham Biosciences) in a final volume of 25 µl. The PCR conditions were 96°C for five minutes, then 35 cycles of 30 seconds at 96°C, 30 seconds at the annealing temperature of the primer (table 1), and one minute at 72°C, followed by one cycle at 72°C for 10 minutes. PCR products were purified using GFX<sup>TM</sup> PCR DNA and the Gel Band purification kit (Amersham Biosciences). All PCRs were carried out in duplicate.

### Direct sequencing

Sequencing was performed by the dideoxy chain termination method using Big Dye technology (Applied Biosystems, Foster City, California, USA). Sequencing primers were the same as those used for PCR. Cycling conditions were as follows: 96°C for five minutes, then 35 cycles of 30 seconds at 94°C, 30 seconds at 51°C, and four minutes at 60°C, followed by one cycle at 60°C for 10 minutes.

The products were purified using an MgCl<sub>2</sub>/ethanol based protocol and run on an ABI 3100 sequencer (AB Applied Biosystems). The results were analysed using 3100 data collection software.

Sequencing was performed twice for each sample to rule out the possibility of PCR fidelity artefacts and was carried out in both directions.

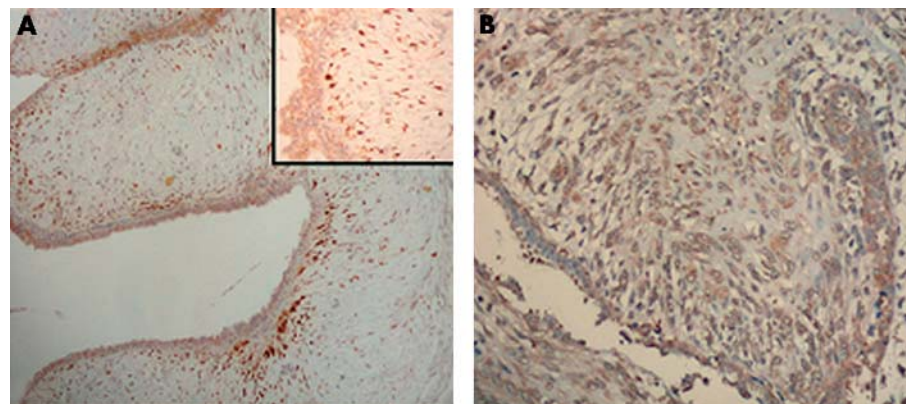
### Statistical analysis

Phyllodes tumours were subclassified into cases with or without c-KIT or PDGFRA expression.

Contingency tables and the  $\chi^2$  test were used applying StatView 5.0 (SAS Institute Inc, Cary, North Carolina, USA) to estimate the correlation between c-KIT or PDGFRA immunoreactivity and the histology of phyllodes tumours. A value was considered significant when  $p < 0.05$ .

### RESULTS

Immunohistochemistry for c-KIT and PDGFRA was performed in 19 phyllodes tumours (table 2). c-KIT stromal expression was found on 12 of the 19 cases (six of the 13 benign cases and all six malignant cases). All six malignant phyllodes tumours had positive stromal expression of c-KIT with strong subepithelial staining (fig 1), compared with six of 13 benign tumours ( $p = 0.02$ ;  $\chi^2$  test; table 3). Epithelial expression of c-KIT was only seen in benign phyllodes tumours (nine of 13;  $p = 0.005$ ;  $\chi^2$  test; table 3). The epithelial component was negative for c-KIT in all malignant cases. PDGFRA showed positive stromal expression in one of the six malignant tumours and one of the 13 benign tumours (not significant). In the epithelial component, all cases were negative for PDGFRA. There was no expression of either c-KIT or PDGFRA in normal breast stromal cells. In contrast, c-KIT was positive in the normal adjacent breast epithelium present in 15 of the 19 cases. Sequencing of exons 9, 11, 13, and 17 of the c-KIT gene only revealed the 2415 C>T alteration in exon 17 in two of the cases (both benign). Analysis of exons 12 and 18 of the PDGFRA gene revealed an intronic insertion, IVS17-50insT, and also the 2866 G>T alteration in the coding region of exon 18 (in one malignant and two benign tumours). This alteration was seen in cases where no PDGFRA expression was detected (table 2). Direct sequencing of 50 unrelated healthy blood donors showed that this alteration could also be found in the normal population (six of the 50 controls had the alteration). None of the activating mutations thus far described were found. Table 3 summarises these results for both benign and malignant tumours.



**Figure 1** (A) c-KIT expression in a malignant phyllodes tumour. Note the subepithelial strong reactivity of stromal cells. The staining pattern can be seen more easily in the inset. (B) Stromal expression of PDGFRA in a malignant phyllodes tumour.

## DISCUSSION

Phyllodes tumours are rare biphasic tumours of the breast that can undergo malignant transformation to sarcoma. Stromal overgrowth is the best known predictor of tumour behaviour. The spindle cell component of these tumours has some similarities with GISTs; namely, both are composed of CD34 positive cells, show a spectrum of behaviour from benign to malignant, and the malignant cases have high rates of recurrence and resistance to conventional chemotherapy.

GISTs are characterised by overexpression of c-KIT. These tumours express constitutively activated c-KIT oncoprotein so that receptor dimerisation and autophosphorylation occur even in the absence of ligand binding.<sup>7-9</sup> c-KIT activating mutations have been described in GISTs and most are found in exons 9, 11, 13, and 17.<sup>9</sup> GISTs that lack mutations in c-KIT have intragenic activating mutations in exons 12 and 18 of PDGFRA. These two pathways seem to be alternative and mutually exclusive.<sup>10</sup> Mutations in either of these tyrosine kinase receptors are important oncogenic mechanisms in GISTs. Moreover, there is currently considerable interest in c-KIT and PDGFRA because of the newly developed drug, imatinib mesylate, which specifically inhibits these tyrosine kinase receptors, and has been used successfully to treat patients with GISTs.

Because of the biological similarities between GISTs and phyllodes tumours and the poor response to the conventional treatment of these tumours, we decided to study c-KIT expression in phyllodes tumours. Two previous reports investigated the expression of c-KIT in a series of phyllodes tumours.<sup>16, 17</sup> Both showed that c-KIT was preferentially expressed in malignant cases. Remarkably, all of our malignant cases were positive for c-KIT and showed preferential expression in the subepithelial stroma. This finding is interesting because alterations in the subepithelial stromal cells in phyllodes tumours are related to malignancy. Increased cellularity, mitotic figures, and p53 immunoreactivity were documented in these areas in malignant phyllodes tumours.<sup>11, 18, 19</sup> The interdependence between stromal and epithelial growth in phyllodes tumours has been discussed in the literature, with some evidence that the epithelial component can be also neoplastic.<sup>18, 20</sup> The loss of c-KIT expression in mammary epithelium is related to malignant breast cancer progression<sup>21</sup>; therefore, the epithelium could

be important for the progression from the benign to the malignant state because in our series almost all of the benign cases had moderate or strong epithelial staining, in contrast to the malignant cases, which were all negative. These findings reinforce the interesting ideas raised by other authors<sup>18</sup> to readdress the issue of the clonal stromal origin of phyllodes tumours. Although in our present study we have only investigated the spindle cell component of the phyllodes tumours, the results obtained indicate that further studies analysing laser capture microdissected epithelial component should be performed.

**"None of the activating mutations described up to now in gastrointestinal stromal tumours was found in our series"**

We did not find activating mutations in our cases of phyllodes tumours. By analysing exons 9, 11, 13, and 17 of the c-KIT gene we only found a silent alteration in exon 17 (2415C>T) in two of the benign cases. Two previous studies also looked at the mutational status of the c-KIT gene and identified two point mutations in exon 11 and one in exon 10 of unknown functional importance.<sup>16, 17</sup> The fact that activating mutations appear to be rare or absent in malignant phyllodes tumours indicates that monotherapy with imatinib mesylate is unlikely to be successful, because malignant tumours must use other growth factor pathways to sustain their growth.<sup>17</sup> However, because clinical trials using tyrosine kinase inhibitors associated with conventional chemotherapy are planned in other tumours that overexpress c-KIT but do not have activating mutations, this approach could be used in the future in malignant phyllodes tumours.<sup>17</sup>

To the best of our knowledge, this is the first description of the immunoexpression and mutational analysis of PDGFRA in phyllodes tumours. We found PDGFRA expression in one of the six malignant cases and in one of the 13 benign cases. We also analysed exons 12 and 18 to check for possible activating mutations (similar to the ones described in GISTs), and found an intronic insertion, IVS17-50T, and an exonic silent alteration, 2866G>T, both in exon 18. These alterations were also found in the normal population, suggesting that they could be population polymorphisms. None of the activating mutations described up to now in GISTs<sup>10</sup> was found in our series.

In conclusion, we have shown that c-KIT is frequently overexpressed in the stroma of phyllodes tumours, predominantly in malignant cases. These results were corroborated by previously published preliminary data.<sup>22</sup> The exact mechanism of this overexpression is unknown because we have found no activating mutations in the c-KIT gene. The PDGFRA does not seem to be an alternative pathway to tumour development because neither expression nor activating mutations were noteworthy. However, the frequent expression of c-KIT in the stroma and the loss of expression in the epithelium in malignant cases raise interesting questions about mechanisms of autocrine/paracrine activation of these receptors and the interdependence of stromal and epithelial interactions in these tumours.

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## Take home messages

- c-KIT is frequently overexpressed in the stroma of phyllodes tumours, predominantly in malignant cases
- However, no activating mutations similar to those described for gastrointestinal stromal tumours were found, so that monotherapy with imatinib mesylate is unlikely to be successful, because malignant tumours must use other growth factor pathways to sustain their growth
- The PDGFRA does not seem to be an alternative pathway to tumour development in phyllodes tumours because expression and activating mutations were similar to the normal population
- The frequent expression of c-KIT in the stroma and the loss of expression in the epithelium in malignant cases raise interesting questions about mechanisms of autocrine/paracrine activation of these receptors and the interdependence of stromal and epithelial interactions in these tumours



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